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THE SEPARATION OF PEPTIDES FROM AMINO ACIDS BY LIGAND-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A rapid ligand-exchange chromatographic method for the separation of α-amino acids from peptides is presented. All α-amino acids, apart from aspartic acid and glutamic acid, are totally excluded from the peptide fraction. Most simple peptides are eluted quantitatively in this fraction, with the exception of some oligopeptides containing carboxy-terminal basic amino acids which are retained on the column. 83% of a tryptic hydrolysate of globin eluted into the peptide fraction. In simple systems, the method can be used to separate peptides from amino acids and to quantitate the peptides at the same time.

INTRODUCTION

This paper describes a method whereby α-amino acids can be rapidly separated from most peptides. The method depends upon the differential affinity of these groups of compounds on ligand-exchange chromatography. The resin which is used is Chelex 100, which is a chelating ion-exchange resin with a high affinity for metal ions, especially the transition metals.

While the metal ions are attached to the resin, they retain their capacity to form coordination complexes. This happens with free α-amino acids, which are therefore retained by the resin. On the other hand, at the pH of this system the stability constants of most peptide–copper complexes are sufficient to remove copper from the resin. In doing so, they form coordination complexes which have a biuret color that can be measured quantitatively. The principle has been used to concentrate amino acids from dilute solutions such as sea-water[1,2] and to separate nucleotides and nucleosides[3]. We have reported a similar method for the study of peptides in human urine[4,5].

The purpose of this report is to describe some modifications of our earlier procedure using a volatile eluent buffer instead of 0.01 *N* sodium borate. We also

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describe here our experience with the technique using pure samples instead of urine in order to study the behavior of known compounds under the test conditions.

We show here that the method can be a useful and rapid means of separating peptides from α-amino acids, and that under some circumstances, the peptides can be measured quantitatively.

MATERIALS AND METHODS

Light absorption measurements were made with either a UV-vis spectrophotometer (Coleman Model 139, Hitachi Perkin-Elmer Corp., Coleman Instruments Division, Maywood, Ill., U.S.A.) or a double-beam scanning UV-vis spectrophotometer (Coleman Model 124) attached to a Model SR recorder (E. H. Sargent & Co., Chicago, Ill., U.S.A.). Molecular extinction coefficients were calculated on the assumption that standards were totally eluted from the column in the first 15.0-ml fraction.

Lyophilates were prepared with a Virtis Model 10-010 automatic freeze dryer (Virtis Co., Gardiner, N.Y., U.S.A.).

Amino acids were quantitated with a modified Technicon analyzer (Technicon, Tarrytown, N.Y., U.S.A.) using Beckman resin PA-28 and a modified gradient elution system[6]. Acid hydrolyses were performed *in vacuo* using 6.0 *N* hydrochloric acid at 110° for 22 h. Samples were lyophilized and reconstituted with 0.2 *N* citrate buffer pH 2.2. Amino acids were dansylated according to the method of Boulton and Bush[7] and subsequently chromatographed according to Morse and Horecker[8].

Standard amino acid mixtures were obtained from two sources, *viz.* Spinco Division, Beckman Instruments, Palo Alto, Calif., U.S.A. and Shandon Products, Consolidated Labs., Chicago Heights, Ill., U.S.A. Individual peptides were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Trypsin-hydrolyzed human whole globin and the β-chain of human globin were furnished by Dr. R. T. Jones, Biochemistry Department, University of Oregon Medical School, Portland, Oreg., U.S.A.

Thin-layer chromatography was prepared using cellulose sheets No. 6064 (Eastman-Kodak Co., Rochester, N.Y., U.S.A.).

Citrate buffer, 0.2 *N*, pH 2.2 was made according to Benson and Patterson[9]. Chelex 100 and Dowex 50W-X8 resins were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Double-distilled water and reagent grade chemicals were used exclusively.

Sodium Chelex 100 resin was washed with 10 bed volumes of water, then added to 20–25 bed volumes of aqueous saturated cupric sulfate solution and stirred for 24 h at 4°. The cupric sulfate solution was decanted and the resin was washed with water until the supernatant showed no copper when tested with sodium diethyldithiocarbamate (DDTC). The resin was stored under water at 4°.

A glass column 0.9 × 24 cm was packed with the resin to a depth of 7 cm. The resin was then equilibrated with 0.1 *N* ammonium hydroxide until the effluent pH was 10.0–10.2. The flow-rate of 0.5–1.0 ml/min was controlled by a stopcock at the bottom of the column. Prior to applying a sample, some effluent was collected for use as a colorimetric blank; absence of copper was verified by adding a crystal of sodium DDTC to an aliquot of this blank.

Except where specified, the samples were dissolved in water, adjusted to pH 10.2–10.8 with concentrated NH_4OH to a total volume of 5.0 ml or less and immediately applied to the resin. The sample was allowed to flow into the resin and elution with 0.1 *N* NH_4OH was started immediately thereafter. As the sample was being applied, the effluent was run into a test tube which contained a few crystals of sodium DDTC. At the first sign of color in the effluent, the next 15.0 ml was collected quantitatively. The optical density of the eluate was measured at 540 nm against the blank. Where specified, copper ions were removed from the ligand eluate by passing it under gravity through a further column consisting of 0.5 × 2.0 cm of sodium Dowex 50W-X8, equilibrated with 0.1 *N* NH_4OH.

RESULTS

The ligand-exchange column retains α-amino acids completely, with the exception of the dicarboxylic acids, which are only partly retained; most peptides with acidic or neutral carboxy-terminal amino acids are eluted in the initial 15 ml. The eluate, which is an ammoniacal solution of peptide–copper complexes, has a biuret-type color, with a maximum light absorption at 500–600 nm, depending upon which peptides are present. A number of non-peptide compounds including uric acid and certain anions including halides[3] and citrate also sequester copper ions and form blue-colored complexes. Strong cationic solutions may also displace copper from the resin, resulting in free copper ions in the eluate[2]; copper ions can be easily removed by passage of the eluate through the Dowex 50W-X8 column.

Free α-amino acids

Beckman amino acid mixture (No. 372220) was adjusted to pH 10.2 and diluted to 0.125 μmoles of each amino acid per milliliter; 5.0 ml of this solution were applied to the ligand column. After the standard elution with 0.1 *N* NH_4OH to 15.0 ml, elution was continued with an additional 20 ml of 0.1 *N* NH_4OH, 20 ml of 1.0 *N* NH_4OH and 20 ml of 3.0 *N* NH_4OH to produce three additional fractions. The four fractions were separately lyophilized, reconstituted and applied to the amino acid analyzer. The recovery of each amino acid is shown in Table I.

As can be seen, only the dicarboxylic acids were eluted in the first fraction. In order to determine whether or not the presence of the citrate buffer in the standard had influenced their elution, an aqueous standard of aspartic and glutamic acids was applied to a Chelex column followed by a standard elution to 15.0 ml. Under these circumstances, approx. 30% each of the two acids appeared in the initial fraction. Citrate buffer, 0.2 *N*, used alone as a sample formed a copper complex that absorbs light strongly at 600 nm.

Dansyl-amino acids

A mixture of 0.65 μmoles of each of the dansyl derivatives of glycine, valine, serine and lysine was dissolved in 0.1 *N* NH_4OH and eluted in the standard manner until the eluate did not fluoresce (15 ml). The eluate was lyophilized, reconstituted with citrate buffer and chromatographed on thin-layer chromatograms alongside some of the original standard. The two samples gave apparently identical chromatograms.

TABLE I

PER CENT RECOVERY OF FREE AMINO ACIDS FROM A LIGAND-EXCHANGE COLUMN AFTER SEQUENTIAL ELUTION WITH DIFFERENT BUFFERS

Amino acid	*Eluent*				*Total recovery (%)*
	0.1 N NH_4OH (15 ml)	*0.1 N NH_4OH (20.0 ml)*	*1.0 N NH_4OH (20.0 ml)*	*3.0 N NH_4OH (20.0 ml)*	
Aspartic acid	94.7	6.4			101.1
Threonine		15.2	75.2		90.4
Serine		22.4	75.2		97.6
Glutamic acid	106.0	4.8			110.8
Proline				75.2	75.2
Glycine		3.2	94.4	13.6	111.2
Alanine		1.6	91.2		92.8
Valine			96.0		96.0
Cystine			93.6		93.6
Methionine			88.8	3.2	92.0
Isoleucine			84.0	9.6	93.6
Leucine			84.8	13.6	98.4
Tyrosine		4.0	82.4		86.4
Phenylalanine			48.0	42.4	90.4
Lysine				53.6	53.6
Histidine				33.6	33.6
Arginine					0

Pure peptide samples

Several experiments were performed using L-leu-ala-gly-ala-phe* (formula weight 477.6) 1.5 mg/ml. The column method gave linear results with increasing quantities of this standard (Fig. 1). The data used to construct the graph shown in Fig. 1 were obtained over a period of several months; the correlation coefficient (r) of this graph is 0.996. The molecular extinction coefficient of the column eluate, calculated from these data, was 201.5 at 540 nm; that for a standard biuret method was 202.5 (ref. 10).

Using a pure peptide solution in this fashion, it was possible to employ the same column repeatedly. Varying the column length from 2.5–17 cm did not change the results.

A similar comparison of the column method with the direct biuret method was performed on 12 individual peptides in aqueous solution. The ligand column results both before and after passage through Dowex 50W-X8 column are shown in Table II and are compared with a standard biuret method.

Simple peptide mixture

A solution of 0.2 μmole/ml of each: glutathione, L-gly-tyr, L-gly-ala and L-leu-gly-phe in 0.2 N citrate buffer was prepared. An aliquot of this solution was chromatographed in the standard manner and subsequently passed through the Dowex 50W-X8 column. Aliquots of both the original solution and the column eluate were

* Standard peptide abbreviations are used throughout.

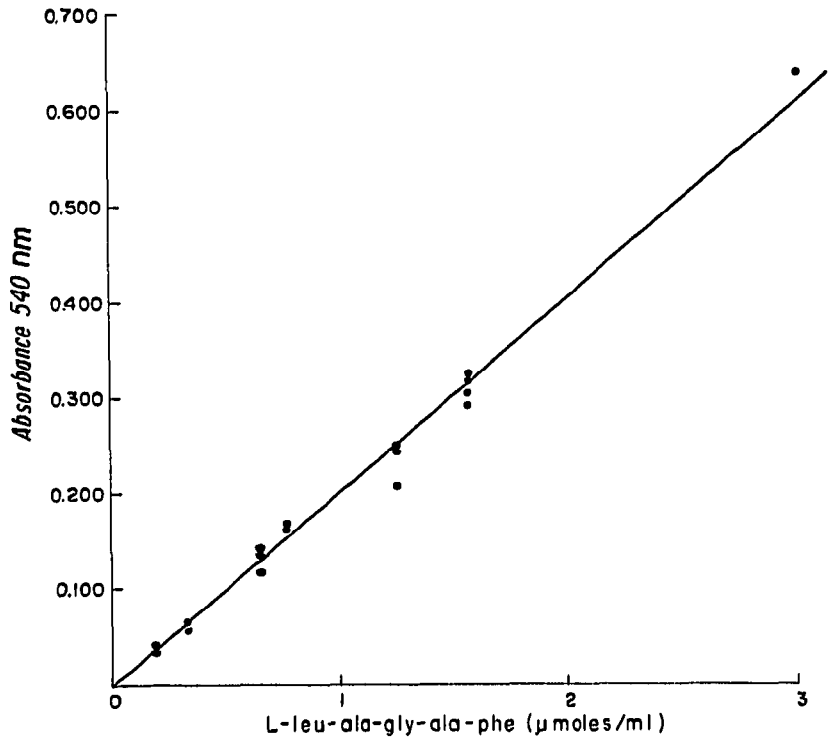

Fig. 1. Cumulative results of a number of experiments using a pentapeptide. The optical density of the ligand-exchange eluate is shown on the ordinate. The regression line has an r value of 0.996.

hydrolyzed, lyophilized, reconstituted with water and run on the amino acid analyzer. Recoveries are shown in Table III.

Trypsin-hydrolyzed globin

A tryptic hydrolysate of human globin was used as a source of a complex mixture of peptides. Various quantities of a solution containing 1.5 mg/ml of the hydrolysate were chromatographed on ligand-exchange columns in the standard manner. The regression line for this series is shown in Fig. 2 ($r = 0.99$). The average molecular extinction was 5279 for the column eluate compared to 2880 for the direct biuret method.

The tryptic hydrolysate was chromatographed on a peptide analyzer before and after passage through the ligand column by B. Brimhall (Department of Biochemistry, University of Oregon Medical School). Several peptides were not detected in the ligand-column eluate, *viz.* tyr-arg (αT-14), gly-his-gly-lys (αT-7), ala-his-gly-lys (βT-7), leu-arg (αT-10), tyr-his (βT-15), val-lys (βT-6), and ala-ala-try-gly-lys (αT-3). The symbols in parentheses refer to the standard nomenclature for peptides derived from the tryptic hydrolysis of human globin.

In order to determine whether extended elution would remove these peptides from the resin 15.00 mg of the tryptic hydrolyzed globin β-chain was chromatographed in the standard fashion and eluted in addition with 25 ml of 0.1 N NH_4OH, 20 ml of

TABLE II

THE MOLECULAR EXTINCTION COEFFICIENTS OF SEVERAL DIFFERENT PEPTIDES OBTAINED BY A STANDARD BIURET METHOD AND BY LIGAND-EXCHANGE CHROMATOGRAPHY

The values for the ligand eluate are shown both before and after passage through a second column of Dowex 50W-X8 to remove free copper ions.

Peptide	*Formula weight*	*Average molecular extinction coefficients*		
		Ligand column		*Direct biuret method*
		Pre-Dowex	*Post-Dowex*	
Leu-phe	278.35	36.46	31.18	18.37
Gly-tyr	238.24	51.78	61.31	17.63
Gly-ala	146.14	43.16	34.58	15.49
Pro-gly	172.18	55.62	48.21	24.11
Leu-gly	188.25	71.44	62.41	50.26
Gly-leu	188.25	35.39	34.64	17.32
Tyr-gly	238.24	45.98	40.34	20.35
Gly-gly-gly-gly	246.22	92.34	102.68	104.89
Glutathione	307.30	245.80		245.80
Gly-gly-gly	189.17	96.86	80.40	53.34
Leu-gly-phe	335.40	140.42	114.48	57.35
Gly-try	261.27	30.31	23.35	
Insulin	5733.00	1221.00	500.00	711.00
Leu-ala-gly-ala-phe	477.60	201.5		202.5

TABLE III

RECOVERY OF AMINO ACIDS AFTER LIGAND-EXCHANGE CHROMATOGRAPHY OF A MIXTURE OF GLUTATHIONE, L-GLY-TYR, L-GLY-ALA AND L-LEU-GLY-PHE

The amino acids were measured after acid hydrolysis of the ligand eluate.

Amino acid	*Amino acid content of hydrolysates of standards*		
	A. Before ligand chromatography (μmoles/ml)	*B. After ligand chromatography*	
		(μmoles/ml)	*(% of A)*
Glutamic acid	0.145	0.138	95.2
Glycine	0.462	0.537	116.2
Alanine	0.139	0.173	124.5
Valine			
Cysteine	0.077	0.045	58.4
Leucine	0.146	0.141	96.6
Tyrosine	0.139	0.169	121.6
Phenylalanine	0.155	0.161	103.9

1.0 *N* NH_4OH, and 20 ml of 3.0 *N* NH_4OH. The four fractions were separately hydrolyzed, lyophilized, reconstituted with citrate buffer and run on the amino acid analyzer. The results are shown in Table IV. The majority of the peptides were present in the first 15-ml fraction.

To check the reproducibility of the method, a tryptic hydrolysate of whole globin was freshly prepared. Four control aliquots of this solution were then acid-

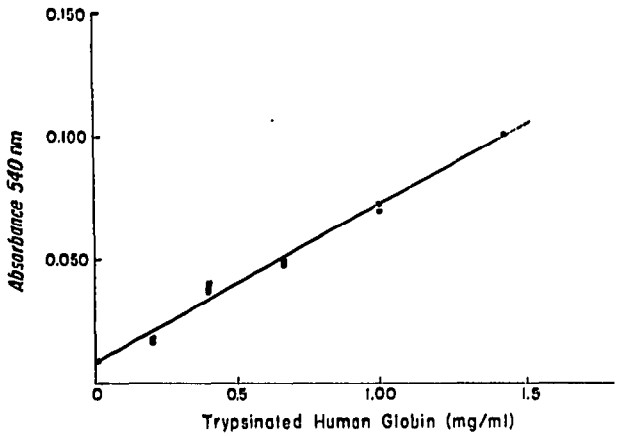

Fig. 2. Cumulative results showing the optical density of the ligand column eluate using trypsinated human globin. The regression line of this graph has an r value of 0.99.

TABLE IV

LIGAND-EXCHANGE CHROMATOGRAPHY OF A TRYPTIC HYDROLYSATE OF HUMAN GLOBIN β-CHAIN

Recovery of amino acids in a series of sequential elutions from a ligand column.

Amino acid	*Recovery from hydrolysed β-chain —Control (μmoles/ml.)*	*Recovery in ligand chromatography fractions (%)*				
		0.1 N NH_4OH (15 ml)	*0.1 N NH_4OH (25 ml)*	*1.0 N NH_4OH (20 ml)*	*3.0 N NH_4OH (20 ml)*	*Total recovery*
Aspartic acid	0.678	92.5	1.0	0.8	0.8	95.1
Threonine	0.341	82.0	2.3	1.2	1.2	86.7
Serine	0.389	93.2	2.4	2.1	3.1	100.8
Proline	0.313	94.5	1.7	1.3	2.1	99.6
Glutamic acid	0.457	97.4	1.5	2.6	2.0	103.5
Glycine	0.666	65.0	2.2	3.0	6.6	76.8
Alanine	0.666	94.2	7.2	4.6	7.0	113.0
Valine	0.422	79.2	1.3	1.3	1.0	82.8
Cystine	0.026	100.0	0.0	0.0	0.0	100.0
Methionine	0.064	35.4	0.0	0.0	0.0	35.4
Leucine	0.690	92.3	1.4	1.0	1.0	95.7
Tyrosine	0.118	75.3	1.1	9.0	2.3	87.7
Phenylalanine	0.332	92.7	1.2	0.8	0.4	85.1
Lysine	0.533	77.5	1.5	2.5	6.8	88.3
Histidine	0.317	126.9	1.3	0.0	0.0	128.2
Arginine	0.141	39.6	2.8	8.5	2.8	53.7

hydrolyzed and six aliquots were put through individual ligand-exchange columns, and acid-hydrolyzed; all the samples were then run on the amino acid analyzer. The results appear in Table V.

TABLE V

LIGAND-EXCHANGE CHROMATOGRAPHY OF SIX ALIQUOTS OF A TRYPTIC HYDROLYSATE OF HUMAN GLOBIN

The amino acids were measured following acid hydrolysis of the ligand eluate. The results marked by an asterisk (*) were invalid due to problems with the amino acid analyzer.

Amino acid	*Average recovery from acid hydrolysis of four 0.50 mg aliquots of trypsinated whole human globin, "A"*	*Recoveries from six 0.50-mg aliquots of whole human globin subsequent to passage through Chelex and acid hydrolysis*						
		1	*2*	*3*	*4*	*5*	*6*	*Average 1–6 as % of "A"*
Aspartic acid	0.239	0.200	0.236	0.223	0.238	0.212	0.238	93.7
Threonine	0.139	0.127	0.130	0.124	0.128	0.121	0.137	92.8
Serine	0.167	0.131	0.148	0.146	0.145	0.133	0.152	85.0
Proline	0.127	0.096	0.118	0.097	*	0.108	0.104	81.9
Glutamic acid	0.196	0.167	0.207	0.146	0.156	0.137	0.155	82.1
Glycine	0.226	0.153	0.178	0.167	0.178	0.137	0.171	72.6
Alanine	0.435	0.333	0.396	0.299	0.338	0.240	0.307	73.1
Valine	0.143	0.139	0.147	0.146	0.154	0.154	0.176	106.3
Half cystine	0.013	*	0.014	0.014	0.014	0.017	0.018	115.4
Methionine	0.026	0.018	0.019	0.016	0.016	0.019	0.020	69.2
Leucine	0.314	0.252	0.274	0.267	0.278	0.258	0.289	85.7
Tyrosine	0.051	0.048	0.048	0.050	0.055	0.042	0.053	96.1
Phenylalanine	0.134	0.109	0.119	0.122	0.121	0.111	0.127	88.1
Lysine	0.207	0.122	0.140	*	0.139	*	*	64.3
Histidine	0.142	0.071	0.111	0.118	0.121	0.109	0.126	76.8
Arginine	0.061	0.048	0.054	0.049	0.048	0.045	0.049	78.7

DISCUSSION

The properties of Chelex 100 make it ideally suited for ligand-exchange chromatography. The active sites on the resin are iminodiacetate groups which form powerful ligand bonds with the transition metals; it seems likely that under the conditions of these experiments the ammoniated copper is ionically bonded to two carboxy groups. Passage of ammoniacal solutions of amino acids and peptides through the resin results in retention of the amino acids with the exception of aspartic acid and glutamic acid, which are partially eluted in the first 15-ml fraction. Failure to retain the two dicarboxylic acids is probably due to their high ionization constants, which are approximately 10^5 greater than those of the monocarboxylic amino acids. The amino acids retained on the resin are probably bound to the copper in typical chelate rings. The peptides which are not retained by the resin are complexed with copper presumably as typical chelates.

Certain peptides in the tryptic hydrolysate which contained two to five residues were retained by the resin. All of these had arginine, lysine or histidine as the carboxy-terminal amino acid; we assume that the presence of an extra free amino group on the carboxy-terminal acid of these peptides permitted them to be handled on the resin in the same manner as free amino acids. Most peptides are eluted quantitatively.

It is possible to prepare the resin in advance and to store it under water in the cold for a period of several weeks. Contrary to Siegel and Degens[1], we have found that exposure of the copper resin to alkaline solutions over pH 10.5 results in the gradual formation of cupric hydroxide and loss of resin selectivity. Before the resin is ready to use, it is necessary to adjust the pH of the effluent to around 10.0–10.5 using 0.1 *N* NH_4OH. This may take some hours and failure to achieve a steady pH in the effluent compromises the results.

The values in Table I show very clearly that not all amino acids behave identically on the resin. The ease with which they are eluted depends to some extent upon the presence of other ions in the sample. The elution of the dicarboxylic amino acids was accelerated by the presence of citrate in the sample.

Our experience with the pentapeptide L-leu-ala-gly-ala-phe, led us to predict that other peptides would behave in a quantitative fashion and would provide results directly comparable with the standard biuret method unless the peptides were retained by the resin. The data which are summarized in Table II show that the results were, indeed, quantitative and reproducible but that they were not usually directly comparable to the standard biuret method.

We have calculated the biuret values as molecular extinction coefficients in order to compare directly the standard biuret method with the results obtained from the column eluent. In doing so, we have made the deliberate assumption that the standard peptides were completely eluted from the column in the initial 15.0-ml fraction. Incomplete elution from the column should have resulted in a lower value by this method. The reason why many of the column values are higher than the standard biuret method is not clear. No copper was detectable in the eluate until after the sample had been applied. The fact that the color yield was usually lower after passage through the Dowex column suggests that some free copper ions were displaced from the resin by the sample and were present in the ligand eluate. These would form a cuprammonium complex with the eluting buffer and would, therefore, contribute to the biuret color at 540 nm.

We used the Dowex column to remove the excess copper ions from the ligand eluate for two reasons. In the first place, it was prudent to remove metals from samples before they were applied to the amino acid analyzer; secondly, we thought that it might improve the correlation between the column and standard biuret methods. The finding that some peptides had higher values after passage through the Dowex column suggests that some rearrangement of the transitional electrons in the ligand bond may have been produced by the conditions in the ion-exchange column.

The resin retards certain oligopeptides, particularly those with lysine, arginine, or histidine at the carboxy-terminal end. Since tryptic hydrolysis of globin produces peptides exclusively with these carboxy-terminal amino acids not all of them were present in the ligand eluate. None of the dipeptides was present and synthetic tyr-lys also was not eluted, whereas lys-tyr could be recovered quantitatively. All of the longer-chain peptides were detected in the ligand eluate, ranging from αT-2 (thr-asn-val-lys) to αT-12, which contains 28 residues. The reason for the apparently low recovery of lysine may be that the free lysine which is present in a tryptic hydrolysate of globin (αT-8, βT-8) is retained by the ligand resin.

It is clear that no method which relies upon ion- or ligand-exchange chromatography will separate all amino acids from all peptides in one maneuver. However,

this simple technique, which separated 95% of free amino acids from 85% of peptides, could be used to advantage in several situations such as the separation of dansylated amino acids from free amino acids, the separation of peptides from amino acids during peptide synthesis or for the preparation of a peptide-rich fraction from biological fluids.

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